ON THE "HEAT COAGULATION" OF PROTEINS. PART IV. THE CONDITIONS CONTROLLING THE AGGLUTINATION OF PROTEINS ALREADY ACTED UPON BY HOT WATER.

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CONTENTS.

Introduction. It has been a matter of common experience since the "heat coagulation" of proteins was first studied, that unless the reaction and salt-content of a protein solution be adjusted within certain limits no precipitate occurs on heating the solution.

Hardy (1) first pointed out that precipitation or coagulation of proteins under the influence of hot water involved two distinct processes, viz. (1) denaturation[1], and (2) subsequent agglutination of the denaturated particles. The same view was afterwards expressed by Pauli and Handovsky (2) (p. 425).

What precisely happens when denaturation of albumen in solution occurs, we are not yet able to say. In cases where agglutination is absent and the solution may remain completely limpid, its viscosity will be found to have considerably increased. This accession of viscosity in an emulsoid colloid indicates, as shown by Hatschek (3), that the volume

[1] We have adopted the term "denaturation" in conformity with the German equivalent "Denaturierung."

of the dispersed phase has increased at the expense of the continuous phase, in other words the colloidal aggregates of protein have taken up more water. At the same time the conditions determining the dispersion of the colloid are altered, for as shown by Hardy (4), the protein can now be completely precipitated by adjusting the reaction so as to render the particles iso-electric with the solution. Small quantities of acid or alkali disperse them and they acquire the property of travelling in an electric field (Hardy (5)). If the amount of acid or alkali present does not exceed what is necessary to obtain a translucent liquid, particles can be seen under the ultra microscope. In the original albumen solution, before heating, the dispersion or solution of the protein *is not dependent upon electric charge* and Michaelis[1], whose important work will be referred to directly, concludes that the properties of the colloidal solution of albumen have by heating been changed from those of an "emulsoid" or hydrophilic colloid to those of a typical suspensoid. Dispersions of denaturated proteins certainly possess the character of suspensoids in so far that the suspension owes its existence to the electrical charge upon the particles, but their other properties, *e.g.* high viscosity and low surface tension, suggest that they are at the same time hydrophile and that they do not fall into either category but form an intermediate group with some of the properties of both.

"Denaturated" egg- or serum-albumen when dispersed in weak alkali, is precipitated by dialysis, by saturating with sodium chloride, potassium chloride or magnesium sulphate (Starke (7)) and by ammonium sulphate in lower concentration than is necessary before heating (Moll (8)). That is to say, these denaturated proteins have acquired some of the characteristics peculiar to globulin. Indeed both Starke and Moll are of opinion that albumen is converted into globulin by heating. Michaelis and Rona ((9) and (10)) have shown that the state of colloidal suspension of serum globulin (eu-globulin) resembles that of heat-denaturated serum-albumen, but these resemblances are concerned with the conditions of dispersion only and do not imply chemical identity between these proteins. Since, according to Abderhalden ((11) and (12)), the crystalline serum-albumen of the horse affords no glycocol on hydrolysis whereas the globulin yields 3·5 % of this amino-acid, these two proteins would appear to possess a fundamentally different chemical constitution.

On the other hand, underlying the change in colloidal conditions produced by heating a solution of albumen, some chemical changes in the

[1] *Physik.-chem. d. Kolloide, Richter-Koranyi's Hdb.* II. p. 391; see also Michaelis and Mostynski (6).

protein molecule have also been traced. Hopkins(13) found that the determination of sulphur in pure egg-albumen which had been coagulated by heat gave uncertain and low results compared with those made upon the original protein precipitated by alcohol, and in the case of crystalline serum-albumen Moll (*loc. cit.*) found that approximately one-third of the sulphur was split off by heat coagulation. The capacity of the protein to combine with acids is also increased on coagulation by hot water (Chick and Martin(14) and (15)). We have sought for evidence of hydrolytic cleavage by determining whether, after the action of hot water, there was any increase in the amount of titratable carboxyl groups as indicated by the formalin method of Sörensen(16). In the case of whole serum this was found to be the case, but with solutions of pure proteins (crystalline egg-albumen, and re-purified pseudoglobulin from horse-serum) we obtained no evidence of hydrolysis when the solutions were heated to 100° C. for two hours in presence of just sufficient alkali to prevent precipitation.

INFLUENCE OF VARIOUS FACTORS UPON THE FINAL STATE OF AGGREGATION OF PROTEIN PARTICLES DENATURATED BY HEATING.

(1) *The influence of reaction.*

A slightly acid reaction is necessary in order to completely precipitate a solution of albumen by heating. The first step towards an interpretation of this fact was made by Hardy((4) and (5)), who examined the electrical properties of a suspension of denaturated egg-white produced by heating a 1 in 10 dilution of this substance, and found that the protein was negatively charged in alkaline solutions and positively charged in acid solution. Further, as the iso-electric point was approached from either side, the stability of the hydrosol diminished, and at the iso-electric point was reduced to zero and precipitation ensued.

Michaelis(17) determined the direction in which unheated serum-albumen wandered in an electric field when the concentration of hydrogen-ions was varied. Different mixtures of primary and secondary phosphates were mixed with the albumen solution to obtain the variations in reaction and precautions were taken to obviate the secondary effects of electrolysis. Michaelis found that the iso-electric point for this protein was considerably on the acid side of neutral, and, in his earlier experiments, determined this point to correspond to a concentration of hydrogen-ions

equal to 10^{-6} normal. From these results he concluded that the "relative acidity" of this protein was between 10^2 and 10^3.

Later Michaelis and Mostynski(6) investigated the iso-electric point of dialysed serum-albumen of the ox both in its natural condition and after denaturation by heat. The reaction of the solution was adjusted by different mixtures either of phosphates or of acetic acid and sodium acetate. Both with natural and denaturated serum-albumen the iso-electric point was found to lie at a concentration of hydrogen-ions ranging from 0·77 to 1×10^{-5} normal. They also determined the optimal acidity for agglutination of the denaturated serum-albumen by adding a small quantity of this to mixtures of acetic acid and sodium acetate in varying proportions. The quickest and most complete agglutination occurred in the mixture with a concentration of hydrogen-ions equal to $\cdot 82 \times 10^{-5}$, which corresponds with the observed value for the iso-electric point. Michaelis and Mostynski agree with Hardy in the interpretation of these results, namely, that at the iso-electric point the charge on the protein aggregates is minimal as they possess an equal tendency to dissociate as positive or negative ions and are not prevented from coming together under the influence of surface tension.

Michaelis and Rona(9) have re-determined the position for optimal flocculation of denaturated serum-albumen with greater accuracy, and have come to the conclusion that in the absence of other electrolytes this occurs at a concentration of hydrogen-ions equal to $\cdot 3 \times 10^{-5}$ normal, *i.e.* somewhat lower than the previously obtained value. The additional observations of Michaelis and Rona on the effect of neutral salts upon agglutination we shall have occasion to refer to later (p. 270). The optimal acidity for flocculation has also been recently studied by Sörensen and Jürgensen(18). They determined the influence of acidity of the solution upon the amount of protein which could be separated by subsequently boiling the solution. They added varying quantities of ·1 N hydrochloric, sulphuric and acetic acid to diluted horse-serum and egg-white, heated the solutions in a water bath at 100° C., filtered them and estimated the nitrogen in the filtrate. The latter was found to be minimal after addition of a certain definite amount of either acid. Excess of the stronger acids interfered with coagulation more readily than excess of acetic acid.

We have made a number of observations during the last three years, the results of which completely confirm the general conclusion arrived at by Michaelis and his collaborators and by Sörensen and Jürgensen. A few of our results are worth mentioning as the experiments were

planned so as to ascertain the range of acidity within which a coagulable protein may be separated from a solution containing it.

Table I illustrates this in the case of diluted horse-serum to which varying amounts of acetic acid had been added. Column 3 shows the range (10–40 c.c. N/100 acid added) over which sufficient aggregation of the denaturated particles occurred to permit of complete separation of the protein by means of a Berkefeld filter. The 4th column shows the range (18–25 c.c. N/100 acid) over which the protein was kept back by filtering through paper.

TABLE I. 10 *c.c. of a* 43 % *solution of horse-serum taken in each case and made up to a total volume of* 80 *c.c. by addition of water and* 1/100 *N acetic acid.*

Amount in c.c. of N/100 acetic acid (or equivalent) added	Appearance of the solution after heating 30 minutes in boiling water	Result of filtering the heated solution through	
		Berkefeld filter	Paper filter
0	Translucent	Filtrate contains protein *	—
5	Opalescent	,, ,, ,,	—
7·5	,,	,, ,, ,,	—
10	,,		98 % of total protein in filtrate.
12·5	Contains precipitate ...		12 % ,, ,, ,,
15	Clear solution, contains precipitate		9 % ,, ,, ,,
18 to 25	,, ,, ,,	Filtrate protein-free	Filtrate protein-free.
27	,, ,, ,,		9 % protein in filtrate.
30	Milky solution, contains precipitate		58 % ,, ,, ,,
40	,, ,, small precipitate		94 % ,, ,, ,,

* Also true after filtration through Doulton filter.

In Table II are set forth the results of a similar experiment with crystalline egg-albumen to which N/100 HCl had been added. Within a small range of acidity the particles aggregate sufficiently to be filtered off by means of filter paper; outside this range of optimum acidity, although not visible to the naked eye, they are for a considerable distance on both sides still sufficiently aggregated to be stopped by a Berkefeld filter. Beyond this second range the particles are too small to be kept back but a proportion of them are visible with the ultra-microscope.

In the experiment detailed in Table III the solution of egg-white was denaturated first and the reaction subsequently adjusted by the addition of N/100 acetic acid. Complete agglutination occurred over a considerable range, but took place more slowly at either end.

TABLE II. *Dialysed* solution of pure egg-albumen*, 1 %. *Total volume*, 10 *c.c.*

No. of exp.	Amount in c.c. of N/100 HCl (or equivalent) added in total volume of 10 c.c.	Appearance of solution after heating 15 mins. in boiling water	Result of filtering the heated solution through paper
1	0	Coagulum in a milky solution	Filtrate contains protein
2	0·5	Coagulum in a clear solution	Filtrate contains trace of protein ... ...
3	1·0†	Coagulum in a clear solution	Filtrate protein free
4	1·5	Opalescent, jelly-like	,, ,, ,,
5	2·0	Opalescent solution	,, ,, ,,
6	5·0	Clear solution ...	Filtrate contains all the protein ... ...

* If it contains any salt the range of agglutination at 100° C. is so much extended that an optimum acidity is difficult to detect.

† Hydrogen-ion concentration $= 10^{-4 \cdot 34}$ normal, determined electrically.

TABLE III. *Denaturated* 3 % *egg-white solution; 5 c.c. made up to a total volume of* 8 *c.c.*

Amount in c.c. of N/100 acetic acid added (or equivalent) in total volume of 8 c.c.	Appearance of solution after standing 24 hours	Result of filtering the solution through	
		paper	Berkefeld filter
0	No agglutination	—	Filtrate contains small amount of protein
1·3	,, ,,	—	—
1·4	,, ,,	—	—
1·5	Almost complete agglutination	Filtrate contains trace of protein	—
1·7	Complete agglutination	Filtrate protein-free	—
2·5			—
3			—
4			—
4·5			—
5	Partial agglutination	—	—
6	,, ,,	—	—
7	Trace of agglutination	—	—

The influence of the nature of the acid. The following experiments, made with diluted horse-serum and hydrochloric, acetic and butyric acids, show, as was to be expected from the observations of Hardy and Michaelis, that the weakest acid gives the widest range within which the denaturated protein can be completely separated by filtration through a paper filter. The results are given in Tables IV, V and VI, and

graphically set forth in Fig. 1. After heating the various solutions to boiling point, the precipitates were caught on a weighed filter paper and weighed after washing and drying at 110° C.

All three acids have an equal effect in inducing agglutination of the protein particles up to the addition of 13–14 c.c. N/100 acid, at which point precipitation becomes complete and a protein-free filtrate is obtained. On further addition of acid, the effect of the three acids

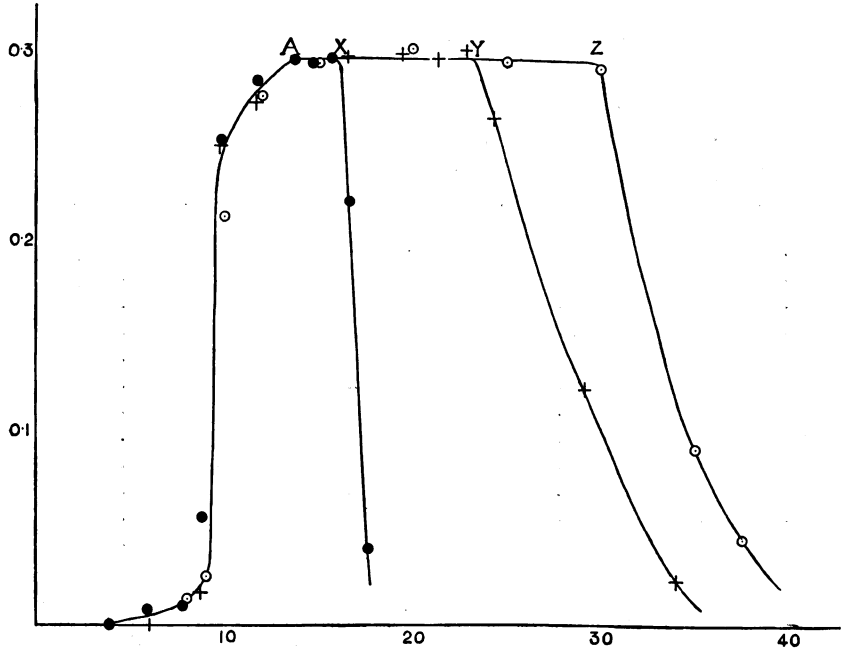

Fig. 1. Amount of protein precipitated which can be separated by a paper filter when 10 c.c. diluted horse serum (42·9 %) are acidified with varying quantities of hydrochloric, acetic and butyric acids and heated for two hours at 100° C. (See Tables IV, V, and VI.) Total volume 80 c.c.

Ordinates = weight of coagulum in grams.
Abscissæ = no. of c.c. N/100 acid added.
—●— = HCl.
—+— = $H_2C_4O_2$.
—o— = $H_4C_8O_2$.

differs. With HCl the zone of complete precipitation extends only over a very small range (from *A* to *X*, Fig. 1), viz. up to the addition of 15·6 c.c. 1/100th normal HCl; beyond this the coherent precipitate becomes gradually less until in the solution containing 19·6 c.c. it is almost entirely dispersed. With acetic acid and butyric acid the range of

complete agglutination continues until the addition of 22·8 c.c. and 30 c.c. 1/100 normal acid respectively (from *A* to *Y* and *Z* respectively, Fig. 1); on adding more acid the amount of the precipitate which can be filtered through paper diminishes, but more gradually than was the case with HCl.

TABLE IV. 10 *c.c. diluted horse-serum* (42·9 %) *acidified with varying quantities of hydrochloric acid and heated for two hours at* 100° *C. Total volume* = 80 *c.c.*

Amount of 1/100 N.HCl added in c.c.	Amount of H_2O added in c.c.	Amount of precipitate in grms.
0	70	0
1·95	68	0
3·9	66	0
5·9	64	·0078
8·8	61	·0100
9·8	60	·2561
11·7	58	·2873
13·7	56	·2988
14·7	55	·2969
15·65	54	·2996
16·6	53	·2238
17·6	52	·0407
19·6	50	small trace

TABLE V. 10 *c.c. diluted horse-serum* (42·9 %) *acidified with varying quantities of acetic acid and heated for two hours at* 100° *C. Total volume* = 80 *c.c.*

Amount of 1/100 N.$C_2H_4O_2$ added in c.c.	Amount of H_2O added in c.c.	Amount of precipitate in grms.
6	64	trace
7·8	62	·0082
8·7	61	·0172
9·7	60	·2529
11·65	58	·2759
14·55	55	·2943
16·5	53	·3003
19·4	50	·3020
21·35	48	·2994
22·8	46·5	·3041
24·3	45	·2684
29·1	40	·1256
34·0	35	·0237

TABLE VI. 10 *c.c. diluted horse-serum* (42·9 %) *acidified with varying quantities of butyric acid and heated for two hours at* 100° *C. Total volume* = 80 *c.c.*

Amount of 1/100 N.$C_4H_8O_2$ added in c.c.	Amount of H_2O added in c.c.	Amount of precipitate in grms.
8	62	·0140
9	61	·0254
10	60	·2158
12	58	·2796
15	55	·2972
20	50	·3052
25	45	·2980
30	40	·2946
35	35	·0936
37·5	32·5	·0459

These results show very clearly the increased working range which can be obtained by using a slightly ionised acid for acidification.

The results of two further experiments with acetic and butyric acids, in which the hydrogen-ion concentration of the various solutions was determined prior to boiling, are detailed in Tables VII and VIII. The observations made in the region of maximum precipitation were not sufficiently numerous to define the optimal hydrogen-ion concentration with exactitude, but the range over which maximal agglutination occurred was almost the same in both instances, *i.e.* ·47 to $1{\cdot}35 \times 10^{-5}$ normal and ·6 to $1{\cdot}74 \times 10^{-5}$ normal when the solutions were acidified with acetic and butyric acids respectively. The mean hydrogen-ion

TABLE VII. 10 *c.c. diluted horse serum** (42·9 %) *heated for* 30 *mins. at* 100° *C. after addition of varying amounts of acetic acid. Total volume* = 80 *c.c.*

Amount of N/100 $C_2H_4O_2$ in c.c.	Amount of H_2O added in c.c.	Amount of the precipitate obtained in grms.	Concentration of H+ in solutions before heating, in terms of normality
0	70	0	$10^{-7.8}$ normal ($0{\cdot}16 \times 10^{-7}$ normal)
5	65	0	$10^{-7.40}$,, (0·4 ,, ,,)
7·5	62·5	0	$10^{-7.27}$,, (0·54 ,, ,,)
10	60	·0074	$10^{-6.86}$,, (1·3 ,, ,,)
12·5	57·5	·2518	$10^{-6.20}$,, (6·4 ,, ,,)
15	55	·2614	$10^{-5.78}$,, (17 ,, ,,)
18	52	·2874	$10^{-5.33}$,, (47 ,, ,,)
22	48	·2912	$10^{-5.05}$,, (89 ,, ,,)
25	45		$10^{-4.87}$,, (135 ,, ,,)
27	43	·2629	$10^{-4.82}$,, (152 ,, ,,)
30	40	·1187	$10^{-4.78}$,, (166 ,, ,,)
40	30	·0160	$10^{-4.49}$,, (324 ,, ,,)

* A different sample from that in Tables IV–VI.

TABLE VIII. 10 *c.c. diluted horse-serum** (42·9 %) *heated for* 30 *mins. at* 100° *C. after addition of varying amounts of butyric acid. Total volume* = 80 *c.c.*

Amount of N/100 $C_4H_8O_2$ in c.c.	Amount of H_2O added in c.c.	Amount of the precipitate obtained in grms.	Concentration of H+ in solutions before heating, in terms of normality
0	70	0	$10^{-7.8}$ normal ($0{\cdot}16 \times 10^{-7}$ normal)
10	60	·0018	$10^{-6.85}$,, (1·4 ,, ,,)
14	56	·2563	$10^{-5.96}$,, (11·0 ,, ,,)
20	50	·2874	$10^{-5.22}$,, (60 ,, ,,)
27	43	·2906	
29	41	·2872	
30	40	·2768	$10^{-4.76}$,, (174 ,, ,,)
40	30	·0448	$10^{-4.63}$,, (229 ,, ,,)
60	10	·0	$10^{-4.33}$,, (468 ,, ,,)

* A different sample from that in Tables IV–VI.

concentration of this range, about 10^{-5} normal, does not differ greatly from that observed by Michaelis and his collaborators for serum-albumen.

The above results support the conclusion of Hardy and Michaelis that the facility with which particles of denaturated protein can be aggregated is conditioned by the concentration of hydrogen-ions in this solution. By working at the high temperature of our experiments, 98° C. to 100° C., the range throughout which agglutination takes place is more extensive than under the conditions of Michaelis' observations.

An explanation of the fact that the iso-electric point for proteins is on the acid side of neutral may be found in the amphoteric nature of the protein molecule. Michaelis (17) considered the position of the iso-electric point to depend upon the relative tendency of the protein to dissociate as acid and base respectively. From theoretical reasoning he concludes that the ratio of hydrogen- to hydroxyl-ions ("relative acidity") at the iso-electric point, is equal to the ratio of the dissociation constants of the protein as acid and base respectively. This was later demonstrated by Michaelis and Davidsohn (19) in the case of amino-benzoic acid where these constants have been experimentally determined. On this view, when sufficient acid is present to reduce ionisation of the carboxyl-group to the same degree as that of the amino-group, the protein becomes iso-electric and aggregates owing to surface tension.

The aggregation of protein particles by electrolytes which will next be discussed seems, however, to be a phenomenon of a different order.

(2) *The influence of neutral salts.*

Pauli and Handovsky (2) studied the influence of a large series of neutral salts upon the coagulation temperature of proteins and (p. 425) attributed the effect of salts in raising the "coagulation temperature" of dialysed ox-serum entirely to their hindering the agglutination of the particles of the denaturated albumen. That this exclusion of any effect of salts upon denaturation-rate is unjustified follows from our own observations ((15), p. 17). Michaelis and Rona (9) found that if serum-albumen were denaturated by heating in alkaline solution, agglutination of the particles took place more readily on addition of acid, if the material had been previously dialysed. The hindering effect of salts was noticeable in the case of NaCl when it was present to the extent of ·06 %, and the process was altogether prevented when the solution

contained more than 0·6 %. In the case of the denaturated proteins of egg-white, on the other hand, agglutination has been shown to be markedly assisted by the presence of neutral salts in small quantities (Hardy (5)).

We have found this to be true also of serum proteins *if the salt is added before heating.* In the present communication we are, however, only concerned with the agglutination of previously denaturated proteins.

The apparent difference in *character* between the influence of neutral salts on the denaturated proteins of serum and egg-white respectively is well seen from the results of experiments (*a*) and (*b*) below.

(*a*) *Horse-serum.*

The solution of denaturated horse-serum was prepared as follows: 10 c.c. diluted serum (43 %), 30 c.c. water and 8 c.c. 1/100th normal acetic acid, the addition of which raised the concentration of hydrogen-ions in the solution to about 10^{-6} normal, were heated for half-an-hour on a water bath at 100° C. A milky solution was obtained which contained no visible particles. This solution was dialysed for two days against tap-water and then diluted with distilled water, until its protein-content was the same as that of the previous experiments (see Tables IV to VIII). This material agglutinated readily in the cold on addition of a suitable amount of acid.

The effect of salts upon agglutination was then studied by comparing velocity of agglutination in presence of a series of different concentrations of acetic acid, both in absence of salts and after addition of various small amounts of sodium chloride and ammonium sulphate.

Agglutination-rate was measured by the time which elapsed after adding the salt to the various solutions before particles appeared which were distinctly visible to the naked eye. The reciprocal of this time, in minutes, was taken as an index of the agglutination-rate. The experiments were made in a thermostat at 37° C. in order that the rate of agglutination might be convenient for study.

The results of four experiments with ·01, ·10, ·2 and ·5 % NaCl respectively are given in Table IX, where column 1 contains the results of a control experiment in which no salt was added, for comparison.

It is seen that concentrations of sodium chloride up to ·1 % are without much effect, but beyond this the rate of agglutination is progressively lowered by the addition of salt and the range of acidity over which agglutination can take place is also curtailed (see Table IX, experiments with ·2 and ·5 % NaCl). The same results are graphically shown in the five curves of Fig. 2, where agglutination-rate is plotted against the amount of acetic acid added. These curves also show very well the effect of salt in necessitating the addition of more acid[1] before the point of optimum acidity for agglutination is reached, or, in other words, in inducing agglutination in solutions otherwise too acid for precipitation.

[1] See also Sörensen and Jürgensen, *loc. cit.* (18), p. 419.

TABLE IX. *5 c.c. of a solution containing 5·3 % of horse-serum, dialysed, diluted to 8 c.c. by addition of water, salt and acid. Temp. = 37° C.*

Amount of N/100 acetic acid (or equivalent) added in c.c.	Rate of agglutination, as measured by the reciprocal of t, the time, in minutes, elapsing after mixing before particles were distinctly visible in the solution, when the concentration of NaCl was				
	0	·01 % (·0017 normal)	·10 % (·017 normal)	·20 % (·034 normal)	·50 % ·085 normal
	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$
0·7	0	0	—	—	—
0·8	2·7	1·7	<0·03	—	—
0·9	—	3·0	0·92	—	—
1·0	—	—	2·63	—	—
1·1	—	—	—	—	—
1·2	2·9	—	3·33	—	—
1·3	—	3·0	3·57	0·03	—
1·4	—	—	3·85	—	—
1·5	3·0	3·3	2·38	0·5	—
1·6	1·09	3·0	1·49	—	—
1·7	0·04	0·1	0·92	0·85	—
1·8	0	—	0·50	—	·07
1·9	—	—	0·15	1·49	·05
2·0	—	—	—	—	·04
2·1	—	—	—	1·72	·03
2·2	—	—	—	—	·02
2·3	—	—	—	1·09	—
2·4	—	—	—	—	<·017
2·5	—	—	—	0·60	—

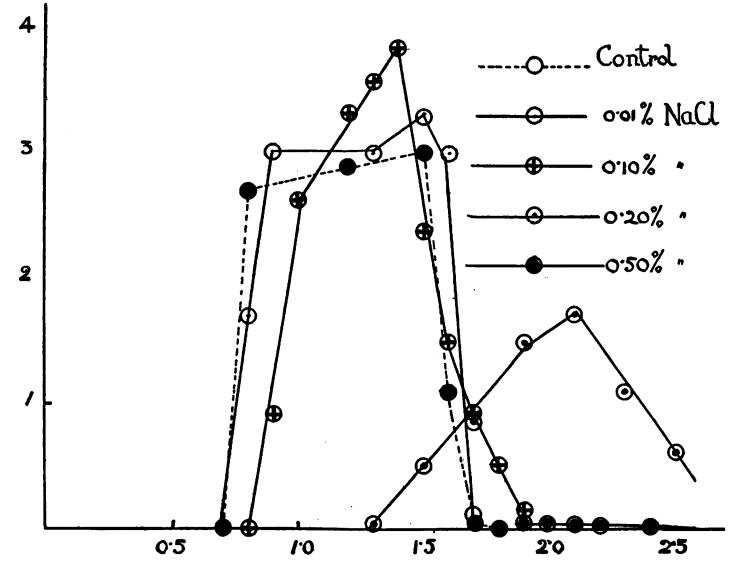

Fig. 2. Agglutination of denaturated horse serum (5·3 %) dialysed, and after addition of NaCl to various concentrations, at 37° C. (See Table IX.)

Ordinates = agglutination-rate, as measured by the reciprocal of the time, in minutes, elapsing before the appearance of visible particles.

Abscissæ = c.c. of N/100 acetic acid (or equivalent) in total volume of 8 c.c.

In Table X and Fig. 3 are given the results of similar experiments, showing effect of ammonium sulphate in concentrations of ·1 and ·5 % respectively. The action of the salt is the same as that of sodium chloride except that the hindering effect upon agglutination is stronger, being already very well marked in a concentration of ·1 %. The

TABLE X. *5 c.c. of solution containing 5·3 % horse-serum, dialysed, diluted to 8 c.c. by addition of water, salt and acid. Temp. = 37° C.*

Amount of N/100 acetic acid (or equivalent) added in c.c.	Rate of agglutination, as measured by the reciprocal of t, the time, in minutes, elapsing after mixing before particles were visible, when the concentration of Am_2SO_4 was		
	0	·1 % (·015 normal)	·5 % (·076 normal)
	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$
0·6	1·09	—	—
0·7	5·88	<0·01	—
0·9	—	0·47	—
1·0	—	1·5	—
1·1	—	2·0	—
1·2	5·88	—	—
1·3	4·00	3·6	—
1·4	1·33	—	—
1·5	0·125	3·0	—
1·7	—	3·0	—
1·9	—	2·0	—
2·0	—	—	·003
2·1	—	1·5	—
2·2	—	—	·003
2·3	—	0·9	—
2·5	—	0·75	·005
2·8	—	0·33	<·003
3·0	—	—	—
3·2	—	0·10	—

alteration of the best agglutinating conditions to those of solutions containing a higher concentration of acid is also more marked in this instance.

In the cases where agglutination was slow in beginning it was usually also incomplete; the solutions containing ·5 % sodium chloride or ammonium sulphate never agglutinated completely during the time they were under observation.

(*b*) *Egg-white.*

A milky solution of denaturated egg-white was employed, similar to that used in investigating the effect of acid upon agglutination (see above, p. 265). 5 c.c. denaturated diluted (1 in 32) egg-white was further diluted to 8 c.c. by addition of various amounts of acid, salt solution and water, so that the solutions contained about ·2 % protein.

A series of solutions were thus prepared with various amounts of acetic acid, and similar series were made up containing in addition 1 and 2 % NaCl as well as the salts naturally occurring in the solution.

Precipitation occurred slowly and completeness or otherwise of the agglutination was observed in the different mixtures after they had stood for 24 hours at room temperature.

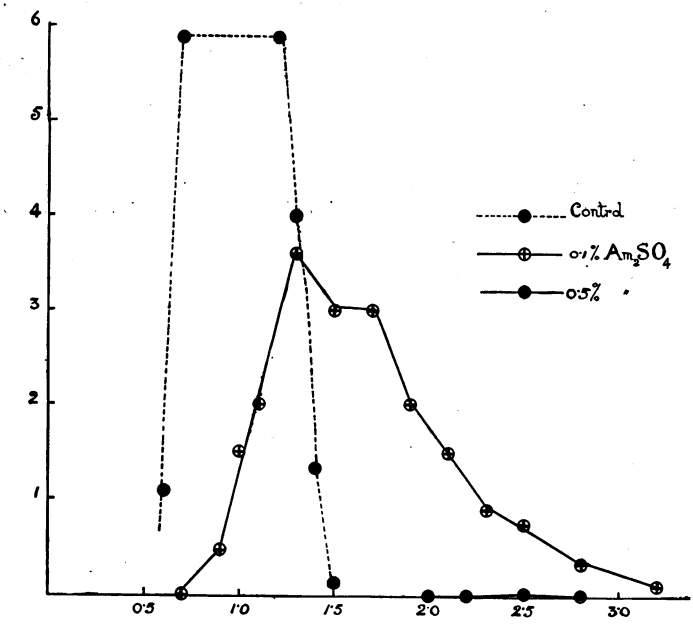

Fig. 3. Agglutination of denaturated horse serum (5·3 %) dialysed, and after addition of Am_2SO_4 to various concentrations, at 37° C. (See Table X.)

Ordinates = agglutination-rate, as measured by the reciprocal of the time, in minutes, elapsing before the appearance of visible particles.

Abscissæ = c.c. of N/100 acetic acid (or equivalent) in a total volume of 8 c.c.

The addition of salt was found to assist agglutination and to very greatly extend, on the acid side, the zone of acidity over which it was complete. In the absence of salt, it was found that complete agglutination took place only in concentration of acetic acid lying between 1·5 c.c. and 5·0 c.c. N/100 acetic acid (or equivalent) in a total volume of 8 c.c. In presence of 1 % NaCl the zone of agglutination extended up to an addition of 3 c.c. N/10 acetic acid, and in the presence of 2 % NaCl up to an addition of 16 c.c. N/10 acetic acid (or equivalent) in the same total volume.

The addition of salts also greatly assists the agglutination of pure crystalline egg-albumen. A dialysed solution of crystals, containing 1 % protein, will only precipitate completely on boiling if the reaction is

carefully adjusted (see Table II above). A 1 % solution of undialysed crystals on the other hand prepared by the method of Hopkins and Pinkus(20) and containing ·3 to ·4 % Am_2SO_4 will coagulate perfectly on boiling over a wide range of acidity (hydrogen-ion concentration $10^{-3\cdot1}$ normal to $10^{-7\cdot4}$ normal, see our previous papers, (14), p. 423 and (15), p. 5).

The effect of ammonium sulphate was also well shown in the following experiment.

A dialysed solution of crystals in its natural reaction on dialysis (hydrogen-ion concentration equal to 10^{-5} normal) was diluted to form a series of solutions containing 1 % protein with various small concentrations of ammonium sulphate; these were then heated for 15 mins. on a water bath at 100° C. In the absence of salt a milky solution was obtained containing a partial coagulum.

Agglutination was rendered almost complete by ·01 % ammonium sulphate. In presence of ·1 % ammonium sulphate complete precipitation took place and the filtrate after filtration through ordinary filter paper was protein-free.

The apparently opposite effect of salts upon the agglutination of egg- and serum-proteins respectively might conceivably have been due to some effect of salts in lowering surface tension at the interface of the colloidal particles and the liquid in the case of serum-protein, and raising it in the case of egg-protein. We endeavoured to test this by seeing whether in the former instance we could obtain evidence of adsorption of salts by the colloid; we found no indication of adsorption in either case.

It seemed likely, therefore, that some light would be thrown upon the different effect of salts upon the agglutination of serum- and egg-proteins by a comparative study of a wider selection of electrolytes and the radicles of which they are composed. Hardy(5) pointed out that a solution of denaturated egg-white offered a satisfactory analogy with certain inorganic colloidal solutions upon which the precipitating action of electrolytes had been more completely investigated. He showed that in acid solutions the protein particles carried a positive electric charge and were sensitive to the anion of the electrolyte added, precipitating power of which was determined by its valency. Similarly in alkaline solutions, where the protein particles are negatively electrified, they display great sensitiveness to the potency of the kation.

The experiments in Table XI are in confirmation of the above.

Diluted egg-white was filtered, heated to about 95° C. on a water bath, precipitated by acidification, filtered, washed free of salts and puddled up with water to make a fine suspension which was at or near the iso-electric point. 5 c.c. of this protein suspension was diluted to 10 c.c. by addition of acid or alkali, salt solution etc., and the effect of electrolytes on agglutination was studied in the case of sodium and barium chlorides and sodium sulphate.

TABLE XI. *5 c.c. of a suspension of denaturated diluted egg-white, at or near the iso-electric point, diluted to* 10 *c.c. with addition of acid or alkali, salt solution, etc.*

+ + + = complete agglutination. + − = almost complete dispersion.
+ + = almost complete agglutination. − = complete dispersion, opalescent solution.
+ = partial agglutination.

Salt added	Concentration, in terms of normality	No. of c.c. N/100 HCl (or equivalent) added in total of 10 c.c.					No addition of either acid or alkali	No. of c.c. N/100 NaOH (or equivalent) added in total of 10 c.c.	
		10	2	1	0·5	0·25		0·5	1·0
Control	...	-	+ −	+	+	+ +	+ + +	−	−
NaCl	·05	...	+	+	+ + +	...	+ + +	+	−
...	·10	+	+ +	+ + +	+ + +	...	+ + +	+	−
Na_2SO_4	·05	+ +	+ + +	+ + +	...	...	+ + +	+	−
$BaCl_2$	·05	+	+	+ +	...	...	+ + +	+ + +	+ + +

The general impression given is that salts assist coagulation. In the case of sodium salts, while the sulphate is much more effective than the chloride on the acid side, both salts exert only a slight effect upon the alkaline side which is equal in the two cases. When the action of the two chlorides is compared it is evident that the action of the barium salt greatly exceeds that of the sodium salt in causing precipitation in the alkaline solution.

Similar experiments with denaturated serum-proteins are given in Table XII. The material consisted of dialysed horse-serum which after diluting and heating was still on the alkaline side of the iso-electric point. In low concentration the effect of salts is exactly analogous to that in the case of egg-proteins, but in concentrations greater than ·01 normal, *all* the salts hindered agglutination.

This difference in the action of electrolytes upon denaturated egg- and serum-proteins disappears when a trivalent anion, such as a citrate, is used. Sodium citrate in low concentration agglutinates particles of both proteins when they are dissolved in acid, and on further addition of the salt both are again dispersed. The salt was prepared so as to be absolutely neutral by titrating citric acid with standard sodium hydroxide, as otherwise the effect is complicated by alteration of the reaction of the solution.

For the experiment set out in Table XIII the material used was a dilute solution of egg-white, boiled, dialysed and further diluted until its protein-content was equal to ·1 %. It was then rendered slightly acid so that the protein particles were dispersed. On addition of sodium

TABLE XII. *Dialysed horse-serum, diluted* 1 *in* 10, *heated to* 90°—100° *C.; 5 c.c. of the milky fluid diluted to* 10 *c.c. with acid or alkali, salt solution and water.*

+ + + = complete agglutination. + − = almost complete dispersion.
+ + = almost complete agglutination. − = complete dispersion.
+ = partial agglutination.

Exp.	Salt added	Concentration in terms of normality	No. of c.c. N/100 HCl (or equivalent) added in total of 10 c.c.									*	No. of c.c. N/100 NaOH (or equivalent) added in total of 10 c.c.				
			10	5·0	3·0	2·0	1·5	1·0	0·75	0·5	0·25		1·0	2·0	3·0	5·0	10
1	Control	...	+	+	+	+	...	+	+ + +	+ + +	+	+	+	+	+	+	−
2	NaCl	·01	..	...	...	...	+	+ + +	+ + +	+	...	...	...	...	...	...	−
3	,,	·05	...	..	+	+	...	+	..	+	...	...	...	...	...	...	...
4	Na_2SO_4	·005	...	...	...	...	+	+ + +	+ + +	+	...	...	...	...	...	...	...
5	,,	·01	...	...	...	...	+ +	+ + +	+ +	+	...	...	...	...	...	...	...
6	,,	·05	...	+	+	+	...	+	...	...	...	...	...	...	...	...	+ −
7	,,	·1	...	...	+	+	...	+	...	...	...	...	...	...	...	...	...
8	$BaCl_2$	·005	...	...	...	+	...	+ +	+ + +	+ + +	...	+	+†	+†	...	...	...
9	,,	·01	...	...	...	...	...	...	+ +	+ +	...	...	...	...	...	...	...

* No addition of either acid or alkali.

† Rather better agglutination than in corresponding tubes without salt.

citrate, agglutination took place when the concentration of the salt was equal to ·0006 normal, but on further addition of the salt, at a concentration between ·002 and ·004 normal, the protein particles were again dispersed and, as was subsequently discovered, bore an electric charge opposite in sign to that originally carried. The concentrations of sodium chloride and sodium sulphate necessary for agglutination was ·10 and ·006 normal respectively. No upper limit for dispersion could be discovered in either case; agglutination remained perfect up to a concentration where precipitation (salting out) of the protein began.

TABLE XIII. *A colloidal solution of denaturated egg-white containing 0·114 % protein was obtained by dispersing the dialysed material by a minimal quantity of (a) acid and (b) alkali; 5 c.c. of the milky solution diluted with water and salt solution to 10 c.c.*

\+ = complete agglutination. − = dispersion.

Concentration of protein %	Salt added	Concentration of salt in terms of normality	Agglutination	Concentration of salt, in terms of normality, necessary to cause: Agglutination	Dispersion
(a) *Original material dispersed by acid.*					
·057	NaCl	·06	−	·10	⋮
		·08	−		
		·10	+		
		·20	+		
		·50	+		
·057	Na_2SO_4	·004	−	·006	⋮
		·006	+		
		·01	+		
		·03	+		
		·05	+		
		·5	+		
·057	Na_3Cit (neutral)	*·0002	−	·0006	·004
		·0004	−		
		·0006	+		
		·0008	+		
		·001	+		
		·002	+		
		·004	−		
		†·006	−		
(b) *Original material dispersed by alkali.*					
·057	NaCl	·01	−	⋮	⋮
		·5	−		
		1·0	−		
		2·5	−		
·057	$CaCl_2$	·01	−	·02	·5
		·02	+		
		·05	+		
		·1	+		
		·2	+		
		·5	−		
		1·0	−		
·057	$La(NO_3)_3$	·0001	−	·0002	⋮
		·0002	+		
		·001	+		
		·01	+		
		·05	+		
·011	$CaCl_2$	·001	−	·03	·5
		·01	−		
		·03	+		
		·05	+		
		·1	+		
		·2	+		
		·5	−		
		1·0	−		
·011	$La(NO_3)_3$	·00001	−	·00005	⋮
		·00005	+		
		·0001	+		
		·001	+		
		·005	+		
		·05	+		
		·1	+		
		·5	+		

* Particles positively charged, H^+ concentration $= 10^{-3.59}$ normal (2580×10^{-7} normal).

† Particles negatively charged, H^+ concentration $= 10^{-6.21}$ normal ($6{\cdot}10 \times 10^{-7}$ normal).

A similar phenomenon was noticed when salts containing multivalent kations were added to alkaline solutions. A colloidal solution of denaturated egg-white in alkaline solution was agglutinated by calcium chloride in concentration of ·02 normal; dispersion, however, again set in at ·5 normal. Although lanthanum nitrate proved a powerful agglutinant, as would be expected from a salt of a trivalent metal, it did not cause dispersion in the concentration employed (see Table XIII).

TABLE XIV. *Horse-serum dialysed.* (*a*) *Heated in solution containing* ·17 *grm. protein and* 1 *c.c.* *N*/100 *NaOH per* 100 *c.c.* (*b*) *Heated in solution containing* ·056 *grm. protein and* 7·7 *c.c.* *N*/100 *HCl per* 100 *c.c.* 2·5 *c.c. of the above solutions diluted to* 5 *c.c. by addition of water and salt solution.*

	Concentration of protein %	Salt added	Concentration of salt in terms of normality	Agglutination[1]	Concentration of salt, in terms of normality, necessary to cause		Sign of electric charge of dispersed particles
					Agglutination	Dispersion	
(*a*)	·085	$La(NO_3)_3$	·00	−			
			·00005	−			
			·00006	+ +			
			·0001	+ +	·00006	·002	
			·0005	+ +			
			·0008	+ +			
			·001	+			
			·002	−			
			·005	−			+
	·085	$CaCl_2$	·005	−			
			·008	+			
			·01	+ +	·008	·02	...
			·02	−			
			·03	−			
	·0085	$CaCl_2$	·00	+ +			−
			·005	+ +			
			·01	+ +			
			·015	+ +	⋮	·02	
			·02	−			0
			·025	−			0
			·03	−			0
(*b*)	·028	Na_3Cit	·0001	−			+
			·0005	−			+
			·0008	+ +			
			·001	+ +	·0008	·01	
			·005	+			
			·01	−			
			·015	−			−
	·028	Na_2SO_4	·001	−			+
			·005	−			+ (slight)
			·01	+ +	·01	·05	
			·05	−			−

[1] + + = agglutination. + = partial agglutination. − = dispersion.

Some comparative experiments on the dispersing power of different salts upon denaturated serum-protein and its dependence upon valency are given in Table XIV. In the case of an acid dispersion the protein (·028 %), was first thrown down, and again dispersed by Na_2SO_4 in concentration of ·01 and ·05 normal respectively. In the case of sodium citrate the corresponding strengths were reduced to ·0008 and ·01 normal. A similar effect was shown by salts containing divalent and trivalent kations upon alkaline dispersions of the same protein, which was dissolved by $CaCl_2$ and $La(NO_3)_3$ in the low concentrations of ·02 and ·002 normal respectively.

The difference in effect of electrolytes upon denaturated serum- and egg-proteins both in acid and alkaline solutions is therefore one of degree only. For the agglutination of both there is an optimum concentration of electrolytes which diminishes as valency rises. Any excess beyond this optimum hinders agglutination and in some cases again disperses the protein. In the case of denaturated serum-proteins the particles are easily dispersed by excess of electrolytes but with egg-albumen this occurred only under the influence of multivalent ions. Hopkins and Savory(21) were evidently dealing with a similar set of phenomena in their work on Bence-Jones protein. They attributed the instability of the heat-coagulum to an extreme sensitiveness to the presence of electrolytes, the "solvent" action of which was especially marked when they consisted of multivalent ions.

Relation of the influence of salts upon agglutination to their effect upon hydrogen-ion concentration. Hardy(22) and Pauli and Handovsky(23) arrived at the conclusion that the hydrogen-ion concentration of acid solutions of protein was diminished by the addition of neutral salts. This conclusion was based on experiments made with indicators, a method which has since been proved to be untrustworthy in the presence of proteins and salts (Michaelis and Rona(24), Sörensen(25)). In our previous papers (14) and (15) we have described experiments confirming the observations of these authors, but showing that when acidity is determined electrically, salts reduced the hydrogen-ion concentration of protein solutions containing a small amount of acid. Further data we have obtained have shown the degree to which this occurs to be dependent upon the valency of the anion.

We have since discovered that there is a parallel action of electrolytes in reducing hydroxyl-ions in alkaline solutions. The experiments were made in solutions containing approximately 1 % pure egg-albumen

crystals, dialysed. The salts employed were NaCl, Na_2SO_4 and $CaCl_2$, in concentrations varying from ·04 to ·13 normal. The results are given in Table XV below and show that the effect is to be attributed mainly to the kation of the salt and to be much increased with rising valency.

TABLE XV. *Reduction of hydroxyl-ion concentration on addition of neutral salts to alkaline protein-containing solutions.*

1 % solution, pure crystalline egg-albumen.

Salt added	Concentration of salt, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Calculated * concentration of hydroxyl-ions, in terms of normality
0	0	$10^{-8.88}$ normal = ($\cdot013 \times 10^{-7}$ normal)	$10^{-5.25}$ normal (56×10^{-7} normal)
KCl	·05	$10^{-8.42}$ normal = ($\cdot038 \times 10^{-7}$ normal)	$10^{-5.72}$ normal (19×10^{-7} normal)
K_2SO_4	·13	$10^{-8.19}$ normal = ($\cdot065 \times 10^{-7}$ normal)	$10^{-5.95}$ normal (11×10^{-7} normal)
$CaCl_2$	·05	$10^{-7.87}$ normal = ($\cdot135 \times 10^{-7}$ normal)	$10^{-6.27}$ normal ($5{\cdot}3 \times 10^{-7}$ normal)

* Dissociation constant of water being taken as $10^{-14.14}$ (see Sörensen[25], p. 161).

The alteration of the reaction of protein-containing solutions by neutral salts is possibly brought about in two ways. The same phenomenon occurs[1] in the case of an amino-acid such as glycocol, where the addition of salt reduces the acidity of a solution containing dilute hydrochloric acid. In this case there is chemical combination between the amino-acid and the salt and definite crystalline compounds have been prepared. Reasoning from analogy, some part of the action described above may therefore be due to decreased hydrolysis of the protein-acid salt or protein-alkali salt, as the case may be, under the influence of a similar chemical union between the protein and the electrolyte. The enhanced effect of salts with di- and trivalent ions is, however, not explained on such an hypothesis.

From analogy with the observations of Whitney and Ober[26], which are referred to on p. 284 below, we are inclined to regard the effect of salts upon hydrogen-ion concentration as the result of selective adsorption of ions by the colloid, the attraction being for that ion bearing a charge of opposite sign. For an adequate explanation of the whole process it would be necessary first of all to possess some knowledge of

[1] Our colleague Dr Atkin kindly determined the hydrogen-ion concentration of solutions of glycocol and small quantities of HCl with and without the addition of NaCl.

the cause of the charge originally carried by the colloidal particles. This is still obscure in the case of inorganic colloids, and although the amphoteric nature of the molecule offers a plausible explanation of the initial charge carried by the protein, it is of no help in explaining the influence of electrolytes upon either the reaction of the solution or the agglutination of the particles.

The question arises as to how far the action of electrolytes in favouring or hindering agglutination, which has been described in the preceding section, may be explained by their effect on reaction. It is evident that a solution, the hydrogen-ion concentration of which is slightly greater or slightly less than that requisite for complete precipitation of the denaturated protein present, may, by the addition of a neutral salt, become adjusted to the degree of acidity appropriate for agglutination. Accordingly a set of experiments similar to those already described above were carried out, with solutions of denaturated egg- and serum- proteins, and the hydrogen-ion concentration was directly measured both before and after addition of the various salts.

The hydrogen-ion determinations were made as described in our previous paper [14] except that in the later experiments we abandoned the convenient device of connecting the two hydrogen cells by means of tubes plugged with filter papers and substituted capillary tubes of 1 mm. bore instead. Mr W. B. Hardy suggested to us that such a method, when dealing with charged colloidal particles, was open to objection, and a series of experiments made to investigate the matter showed that in the case of protein solutions a slight error was introduced. We are, however, satisfied that the magnitude of any error due to this practice is not such as to materially affect our previously published results.

Egg-white. The results of a set of experiments with denaturated egg-white are set out in Table XVI. The material was exactly similar to that employed above (Table XI) and was at or near the iso-electric point. In the absence of salts the agglutination-zone was found to be at a concentration of hydrogen-ions between 3 and 900×10^{-7} normal. Addition of Na_2SO_4 to a concentration of ·005 and ·05 normal extended the range on the acid side to a hydrogen-ion concentration of 1400 and $20{,}000 \times 10^{-7}$ normal respectively. At the same time the presence of the salt greatly reduced the hydrogen-ion concentration from what it would have been in its absence. Similarly, addition of barium chloride to a concentration of ·005 normal lowered the concentration of hydroxyl-ions and increased the range of agglutination on the alkaline side of the iso-electric point.

Horse-serum. The action of sodium sulphate in reducing hydrogen-ion concentration in solutions containing particles of denaturated serum-protein is similar to what occurs in the case of egg-proteins and, when

TABLE XVI. *A solution of diluted egg-white, filtered, heated, precipitated by addition of acid and washed free of salts, puddled up with water to make a fine suspension; 5 c.c. of the suspension diluted to 10 c.c. by the addition of acid or alkali, salt solution, water.*

Exp.	Salt added	Concentration of salt, in terms of normality	No. of c.c. N/100 HCl (or equivalent) added in total of 10 c.c.	No. of c.c. N/100 NaOH (or equivalent) added in total of 10 c.c.	Hydrogen-ion concentration, in terms of normality	Degree of agglutination
1	—	—	—	·5	$10^{-7.39}$ normal ($0{\cdot}405 \times 10^{-7}$ normal)	Dispersed.
2	—	—	—	·25	$10^{-6.75}$ normal ($1{\cdot}77 \times 10^{-7}$ normal)	,,
3	—	—	—	·17	$10^{-6.53}$ normal ($3{\cdot}0 \times 10^{-7}$ normal)	,,
4	—	—	0·00	·00	$10^{-5.54}$ normal ($28{\cdot}9 \times 10^{-7}$ normal)	Agglutinated.
5	—	—	0·08	—	$10^{-4.03}$ normal (934×10^{-7} normal)	Almost completely agglutinated.
6	—	—	0·17	—	$10^{-3.89}$ normal (1280×10^{-7} normal)	Slightly agglutinated.
7	—	—	0·25	—	$10^{-3.69}$ normal (2033×10^{-7} normal)	Dispersed.
8	—	—	0·50	—	$10^{-3.28}$ normal (5190×10^{-7} normal)	,,
9	—	—	1·0	—	$10^{-3.19}$ normal (6460×10^{-7} normal)	,,
10	Na_2SO_4	·005	0·5	—	$10^{-4.13}$ normal (736×10^{-7} normal)	Agglutinated.
11	,,	,,	0·75	—	$10^{-3.85}$ normal (1407×10^{-7} normal)	Almost completely agglutinated.
12	,,	,,	1·0	—	—	Almost completely dispersed.
13	,,	·05	—	·17	$10^{-6.78}$ normal ($1{\cdot}6 \times 10^{-7}$ normal)	Almost completely agglutinated.
14	,,	,,	—	·25	$10^{-6.47}$ normal ($3{\cdot}4 \times 10^{-7}$ normal)	,, ,, ,,
15	,,	,,	1·0	—	$10^{-4.00}$ normal (1000×10^{-7} normal)	Agglutinated.
16	,,	,,	5·0	—	$10^{-2.70}$ normal ($20{,}000 \times 10^{-7}$ normal)	,,
17	$BaCl_2$	·005	—	·5	$10^{-7.064}$ normal ($0{\cdot}86 \times 10^{-7}$ normal)	,,

present in low concentration, agglutination may be assisted in acid solution, see Exp. 8, Table XVII. On the other hand, when this salt is present to the extent of ·05 normal, agglutination is prevented *even when the hydrogen-ion concentration may be the optimum for precipitation*, see Exp. 11, Table XVII.

Whatever may be the nature of the process involved in the phenomenon, it is clear that the change in reaction of protein solutions caused by electrolytes is inadequate to explain the influence of the latter upon agglutination.

The interpretation of the action of electrolytes in agglutinating and dispersing protein particles. At first sight it appears puzzling that a colloidal solution of denaturated protein should be destroyed by a certain concentration of an electrolyte and that solution should again take place

TABLE XVII. *Dialysed horse-serum, diluted* 1 *in* 10, *heated to about* 95° *C.; 5 c.c. milky fluid diluted to* 10 *c.c. by addition of acid or alkali, water, salt solution.*

Exp.	Nature of salt added	Concentration of salt, in terms of normality	C.c. N/100 HCl (or equivalent) added in total vol. of 10 c.c.	Hydrogen-ion concentration, in terms of normality	Degree of agglutination
1	—	—	0	$10^{-7.08}$ normal ($\cdot 83 \times 10^{-7}$ normal)	Dispersed.
2	—	—	0·5	$10^{-6.36}$ normal ($4{\cdot}35 \times 10^{-7}$ normal)	,,
3	—	—	0·6	$10^{-6.08}$ normal ($8{\cdot}22 \times 10^{-7}$ normal)	Complete agglutination.
4	—	—	0·75	$10^{-5.51}$ normal ($30{\cdot}7 \times 10^{-7}$ normal)	,, ,,
5	—	—	0·85	$10^{-4.54}$ normal (287×10^{-7} normal)	Dispersed.
6	—	—	1·0	$10^{-4.09}$ normal (803×10^{-7} normal)	,,
7	Na_2SO_4	·005	0·5	$10^{-6.56}$ normal ($2{\cdot}77 \times 10^{-7}$ normal)	,,
8	,,	,,	1·0	$10^{-5.20}$ normal ($63{\cdot}5 \times 10^{-7}$ normal)	Complete agglutination.
9	,,	·05	0	$10^{-7.00}$ normal ($1{\cdot}00 \times 10^{-7}$ normal)	Dispersed.
10	,,	,,	0·5	$10^{-6.42}$ normal ($3{\cdot}81 \times 10^{-7}$ normal)	,,
11	,,	,,	1·0	$10^{-5.51}$ normal ($30{\cdot}6 \times 10^{-7}$ normal)	,,

on further addition of the same salt. Precipitation of inorganic colloidal solutions by electrolytes is apparently due to their discharge following the adsorption on the particles of that ion of the electrolyte which bears a charge opposite in sign to their own. Linder and Picton (27) found that when the negatively charged particles of arsenic trisulphide were thrown out of their colloidal solution by the addition of barium chloride, barium was present in the sulphide precipitate. Whitney and Ober (26) confirmed the observation of Linder and Picton and showed that an exactly

equivalent amount of chlorine remained behind in the solution as free acid so that the reaction of the solution was materially altered. Lewis (28) obtained evidence indicating that, in the case of an emulsion of petroleum oil precipitated with barium chloride, barium was adsorbed on the particles of oil in excess of chlorine. In all these cases agglutination is associated with discharge of the particles.

If the adsorption of ions were to continue in excess of the amount required to discharge the particles, they would take on the opposite charge with the corresponding lowering of surface action. The particles would then again repel one another and dispersion would ensue. This has actually been shown to occur by Burton (29), who found that colloidal copper particles, which carry a positive charge, will migrate to the anode after addition of excess of potassium phosphate to the solution. Reasoning from this analogy, the explanation, for example, of the behaviour of egg-white proteins towards sodium citrate in acid solution, would be that the negatively charged anions when present in minimal concentration are able to discharge the positive charge carried by the protein particles; if, however, the concentration of the citrate is increased, the transaction is overdone owing to the powerful charge carried by the trivalent anion, and the protein, first becoming discharged, is afterwards re-charged with a sign opposite to that originally held[1]. The difference between denaturated serum- and egg-proteins then becomes one of degree and not of kind. Dispersed particles of the latter are readily discharged but with difficulty overcharged; in the case of the former the tendency to become overcharged is so great that electrically neutral particles are obtained with difficulty if electrolytes are present in more than minimal concentration, especially when these are composed of multivalent ions.

The colloidal particles of proteins would seem to be extraordinarily sensitive to this action of electrolytes and to display a quite peculiar tendency to adsorb ions. To test this hypothesis it was necessary to compare the sign of the electric charge on protein particles before and after dispersion by salts. The direction of migration in an electric field was observed under a microscope with a dark ground illumination. For this purpose the degree of dispersion must not be too great or the particles become invisible with the ultra-microscope. The liquid was placed in a shallow glass cell built upon a microscope slide

[1] The observations of Mines (30) are also of the same order; he has shown that the red blood-cells of *Scyllium canicula* are clumped by low concentrations of cerium chloride and again dispersed when the concentration of the salt is raised, the cells acquiring an electric charge of reverse sign to that originally carried.

(Fig. 4). The sides of the cell were formed by two slips of cover-glass ·3 mm. in thickness cemented upon the slide with balsam. The ends of the cells were composed of two pieces of platinum, of the same thickness, similarly cemented on to the slide. The roof of the cell was formed by a thick cover-glass. One of the pieces of platinum was a little shorter than the other so as to permit of a small exit channel for the excess of liquid when the cover-glass was placed in position. To each of the pieces of platinum foil a spiral of platinum wire was welded

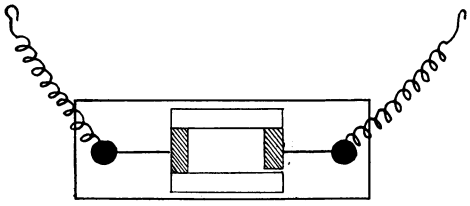

Fig. 4.

which served to convey the current from the accumulators. The electrodes were 2 cm. apart and the difference of potential employed was 8 volts. In using the method, in order to avoid the disturbing effects of electrolysis it is essential that the circuit be only momentarily closed whilst making the observations. The current is then reversed and the observation repeated. If it is desired to measure the velocity of movement this can be done by the aid of an ocular micrometer previously standardised for the particular optical combination used. The dispersion must be a very dilute one or too many particles will be present.

In using this method the particles observed must be situated some distance from the surface of the glass cover or slide, as in the neighbourhood of the glass the liquid moves under the influence of electric endosmose and of course carries the particles with it. With the electric field we employed, 4 volts per cm., this effect was confined to a depth of ·05 mm. from either surface. Another limitation of the method is that if the concentration of electrolyte is above a certain limit, gas bubbles arise at the electrodes and cause movements of the particles. The range of concentration of electrolyte with which it is possible to work without formation of gas is considerably extended by covering the electrode with platinum black.

In the case of serum, it is seen from Table XIV that dispersion caused by sodium citrate and sodium sulphate in acid solution was accompanied by a change of sign in the charge carried by the protein particles from

positive to negative. That this effect is not dependent upon reduction in hydrogen-ion concentration is seen in Table XVIII, where particles of denaturated serum-protein dispersed by Na_2SO_4 carry a negative charge in a solution which is acid of the iso-electric point.

In alkaline solution the dispersed particles with their − charge were agglutinated and afterwards dispersed by $CaCl_2$ and $La(NO_3)_3$ but the possession of a + charge was only demonstrated in the case of the latter salt. The colloidal solution with $CaCl_2$ is difficult to explain as we were not able to demonstrate the existence of any charge on the dispersed particles (see Table XIV). The solution of denaturated egg-white by $CaCl_2$ solution is also anomalous, seeing that no dispersion was obtained

TABLE XVIII. *Influence of Na_2SO_4 upon the charge carried by and agglutination of particles of denaturated serum-protein and upon the hydrogen-ion concentration of the solution.*

Exp.	Salt added	Concentration of salt added, in terms of normality	C.c. N/100 HCl (or equivalent) added in total vol. of 10 c.c.	Concentration of H+ ions, in terms of normality	Sign of electric charge on particles	Degree of agglutination
1	—	—	·1	$10^{-6.42}$ normal ($3{\cdot}77 \times 10^{-7}$ normal)	...	Partial agglutination.
2	—	—	·15	$10^{-6.23}$ normal ($5{\cdot}86 \times 10^{-7}$ normal)	...	Almost complete agglutination.
3	—	—	·20	$10^{-4.51}$ normal (310×10^{-7} normal)	...	Complete agglutination.
4	—	—	·50	...	+	Dispersed.
5	—	—	·70	$10^{-3.24}$ normal (5780×10^{-7} normal)	+	,,
6	Na_2SO_4	·03	·30	$10^{-5.45}$ normal (352×10^{-7} normal)	...	Almost complete agglutination.
6	,,	·07	·70	$10^{-4.01}$ normal (970×10^{-7} normal)	−	Dispersed.

with $La(NO_3)_3$ which from all analogy should have had a more powerful effect. In the case of the dispersion by $CaCl_2$ the concentration of electrolyte required was too great to make any satisfactory observations upon the charge carried. A change of sign in the latter was however demonstrated in the case of egg-white and sodium citrate. The particles were previously dispersed in acid solution with a positive charge, and on addition of sodium citrate were at first precipitated. With higher concentration of the salt the particles were again dispersed, this time bearing a negative charge (see Table XIII).

We have not been able to determine wherein lies the cause of the extra tendency to overcharge in the case of serum-proteins. It is conceivable that the charge carried by the particles may be small in

amount and easily overbalanced or it need only be very small in order to keep the particles dispersed from one another. On the other hand, serum-proteins may be able to adsorb ions more readily than egg-proteins.

The sensitiveness of agglutination to minute variations in reaction and salt-content explains why it is so difficult to entirely separate serum-protein by heating the solution. It is practically impossible to perfectly adjust the reaction so that the whole of the protein is precipitated. The finding of more or less non-coagulated protein serum by Chabrié(31), Howell(32), and others is no doubt to be explained in this way.

(3) *Influence of temperature.*

(*a*) *Horse-serum.* The same solution of denaturated serum was used as in the experiments made to investigate the effect of salts given in Tables IX and X.

Convenient material for study, *i.e.* one which failed to agglutinate at laboratory temperatures and did so at a suitable rate for study at higher temperatures, was the following: a 3·3 % suspension of denaturated horse-serum, dialysed, containing in 100 c.c. 3·7 c.c. N/10 acetic acid and 1 gram NaCl in Exp. 1, and 4·4 c.c. N/10 acetic acid and ·5 grm. Am_2SO_4 in Exp. 2. Tubes containing these mixtures were placed in thermostats at different constant temperatures and agglutination-rate was measured by observing the time which elapsed before visible particles appeared.

The results of Exps. 1 and 2, set forth in Tables XIX and XX, show that there is a critical temperature below which agglutination either does not take place at all or so slowly as to be negligible for our purpose. Immediately above this point, temperature exerts a very great effect on agglutination-rate; on raising the temperature still further the influence of a definite rise in temperature becomes gradually less and less until, at temperatures far removed (30°–40° C.) from the critical temperature, the effect becomes consistent, and the temperature coefficient of agglutination settles down to a value of about 2·5 per 10° C. rise of temperature. This is shown graphically in Fig. 5, where logarithms[1] of agglutination-rate (as measured by the reciprocals of the time, in minutes, before visible particles could be detected) are plotted against temperature. In the neighbourhood of the critical temperature the curves (*a*) Exp. 1

[1] Logarithms are plotted, as the effect in the region of the critical point is so enormous that the numbers themselves could only have been set out on a very small scale.

and (*b*) Exp. 2, rise steeply; above temperatures of about 60° C. in either case the curves tend to form straight lines, showing that the effect of temperature at this point is becoming consistent.

As there is no reason to suppose that temperature would greatly modify either the surface tension between particles and fluid or the charge on the particles, we suggest the following explanation to account for these facts. The surface energy which brings about aggregation is

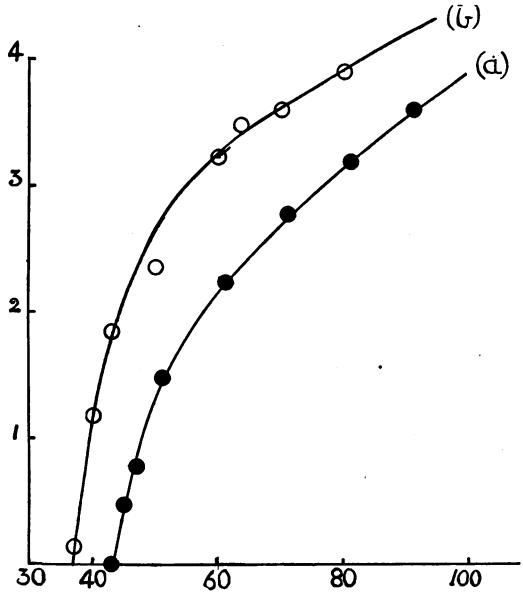

Fig. 5. Effect of temperature upon agglutination of "denaturated" diluted serum (3·3 %) containing:

(*a*) 1·0 grm. NaCl and 3·7 c.c. N/10 acetic acid in 100 c.c.

(*b*) 0·5 grm. Am_2SO_4 and 4·4 c.c. N/10 acetic acid in 100 c.c.

Ordinates = $\log_{10}$ (agglutination-rate × 10^3). See Tables XIX and XX.

Abscissæ = temperature, degrees Centigrade.

potential until the particles come nearly into juxtaposition. They are brought into the necessary close relationship with one another by their own movement or the movement of the fluid, the former, or Brownian movement, being the more important in the case of very fine particles. If the particles carry an electric charge, their velocity of movement will be diminished as soon as they are brought within the range of the electrical repulsion, and unless the momentum is adequate to counteract this repulsion, the particles will never arrive sufficiently close to one another for the effect of surface energy to be manifested. On this view

TABLE XIX. *Exp.* 1. *A suspension of denaturated diluted serum,* 3·3 %, *dialysed, containing in addition* 3·7 *c.c.* 1/10 *N acetic acid in* 100 *c.c. and NaCl to a concentration of* 1 %.

Temperature, °C.	Time elapsing after mixing before particles were visible, mins. = t	Agglutination-rate, as measured by I/t	Increase in agglutination-rate for a rise in temperature of 10° C.
43	780	0·001	...
45	390	0·003	
47	180	0·006	...
51	36	0·03	
61	6	0·17	
71	1·62	0·61	... ... 3·7
81	0·65	1·54	... ... 2·5
91	0·25	4·0	... ... 2·6

TABLE XX. *Exp.* 2. *A suspension of denaturated diluted serum,* 3·3 %, *dialysed, containing in addition* 4·4 *c.c.* 1/10 *N acetic acid in* 100 *c.c. and* Am_2SO_4 *to a concentration of* ·5 %.

Temperature, °C.	Time elapsing, after mixing before particles were visible, mins. = t	Agglutination-rate, as measured by I/t	Increase in agglutination-rate for a rise of 10° C.
37	>360 and <1020	...	...
40	67	0·015	
43	14	0·071	...
50	4·2	0·23	
60	0·58	1·72	
63·5	0·33	3·00	... ... 2·3
70	0·25	4·00	
80	0·125	8·00	... ... 2·0

there exists a "critical velocity" necessary for agglutination and, as the velocity of the particles is a function of temperature, an explanation is afforded of the observed critical temperature below which agglutination does not occur.

We interpret the high temperature coefficient just above the critical temperature and the subsequent falling off of the temperature effect as follows: At any given temperature we assume the degree of intrinsic energy possessed by the protein particles to be distributed among them in the manner illustrated in Fig. 6, which represents the normal frequency curve. Suppose the curve I to express the distribution of momentum among the particles at temperature t_1; if A and B indicate any two values of this intrinsic energy, the volume of the rectangle $Aa\ bB$, compared with the volume included between the curve and the base line, represents the proportion of particles possessing a kinetic

energy which is greater than A and less than B. If now C represents the critical momentum which gives rise to a velocity sufficient to overcome the existing forces of repulsion between particles, it is seen that at temperature t_1 the material in question will contain practically no particles possessing the required critical velocity. Agglutination, therefore, will only occur with extreme slowness.

As the temperature is raised and the mean value of the particular energy is consistently increased, the distribution curve, while maintaining the same form, will be moved as a whole towards the right. Suppose curve II is the frequency curve at a higher temperature t_2. At this temperature the number of particles, N, which possess more than the critical velocity, represented by the volume CcD, will be relatively important and agglutination will now begin to be apparent.

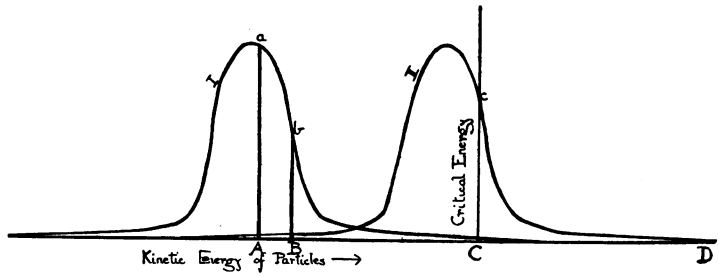

Fig. 6. Illustrating the distribution of kinetic energy among protein particles in colloidal solution at different temperatures.
Ordinates = distribution of particles possessing kinetic energy corresponding to abscissæ.

A further rise in temperature from this point onwards will cause a disproportionate increase in the value of N, for while with small difference in temperature, the curve as a whole may be displaced only a short distance to the right, the area CcD, which represents the value of N, will be relatively much increased. In the range of temperature immediately above the critical point the effect of temperature will therefore appear to be greatly enhanced.

At higher temperatures still, where the crest of the frequency curve has reached a point to the right of C, an increasing majority of the particles will possess more than the critical momentum, until, at temperatures at which all the particles possess a velocity greater than the "critical velocity," the effect of temperature will merely increase the mean value of the momentum of the particles, and will settle down to the conventional value.

THE SUPPOSED CONVERSION OF ALBUMEN INTO GLOBULIN BY HEATING IN SLIGHTLY ALKALINE SOLUTION.

We have already mentioned (p. 262) the conclusion of Starke(7), viz. that on heating a solution of egg-albumen in its natural slightly alkaline reaction, the whole of the albumen was converted into globulin. Moll(8) arrived at a similar conclusion from his experiments with serum-albumen and showed that the precipitability of serum by means of Am_2SO_4 became altered, inasmuch as the euglobulin and pseudoglobulin fractions (Pick) were increased at the expense of the albumen. Starke based his interpretation upon the facts that egg-albumen so treated was precipitated by dialysis, by dilution and by acidification, and could be dissolved readily by slight excess of acid or alkali. It could be salted out by saturation with sodium chloride, potassium chloride or magnesium sulphate and, like globulin, contained less sulphur than the original albumen. Starke regarded the material as distinct from "denaturated" albumen because on heating a suspension of his supposed globulin in the presence of salts (NaCl, $CaCl_2$ or $MgSO_4$) it underwent "heat coagulation" and became converted into a firm and coherent clot which, in contradistinction to his manufactured globulin, he found to be insoluble in ·05 % sodium carbonate or in weak acid.

The observations of Starke and Moll amount to this. If a solution of albumen be heated to 60°–85° C. in presence of a small concentration of alkali, the albumen is changed into a body possessing the "solubilities" and some other properties of globulin. This body is precipitated from the solution by adjusting the reaction so as to be slightly acid (iso-electric point). On heating a similar solution of albumen rendered just acid a coherent precipitate is obtained which appears to be insoluble in water and dilute acids or alkali. The difference depends upon whether the reaction be made acid before or after heating. There is no doubt about the accuracy of the observations, but we do not believe the facts warrant the interpretation put upon them by these authors. The difference in the ease with which the two precipitates can be dissolved (or, more correctly, dispersed) by acids and alkali is, we believe, bound up with the different degree of aggregation of the particles in the two precipitates, and we see no reason to suppose that Starke's "globulin" is other than "heat-denaturated" albumen. We find that the firm coherent precipitate of egg-albumen or serum-albumen formed by boiling in acid solution can be dispersed completely although slowly by alkali

or acid, the amount required depending on the concentration of the protein (1/100th normal alkali for a concentration of ·5 % protein), if the particles are first mechanically separated, and a few days allowed for the process to take place.

It seems to be merely the differing degree of cohesion of the particles which determines the different solubility of the material. From what has gone before it is clear that the dispersion of denaturated albumen depends upon the possession of an electric charge, whence it follows that the difficulty in dispersing the particles of a curdy, coherent precipitate is due to the difficulty of charging them.

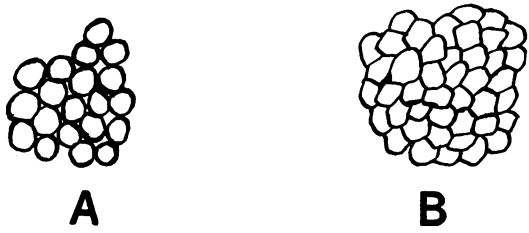

Fig. 7.

A and *B*, Fig. 7, represent the particles in a fragment of loosely agglutinated and coherent cheesy precipitate of denaturated protein respectively, as seen under the ultra-microscope. We suggest that the reason *A* disperses readily and *B* only slowly on addition of weak acid or alkali is that, in the former case, the charge is situated on the surface of the nearly discrete particles, whereas in the latter case the coherent aggregate is charged as a whole. In *A* a small charge is sufficient to tear the particles asunder, whereas in *B* any disruptive action or effect on surface tension is limited to the superficial particles, and even here operates at great mechanical disadvantage.

SUMMARY.

1. As shown by Hardy, Michaelis and others, dispersion of denaturated protein by small amounts of acid or alkali is due to the electric charge given to the particles. If this electric charge is neutralised, and the proteins become iso-electric with the solution, agglutination occurs.

2. In common with other observers, we find that reaction of the solution is the principal factor in determining the degree of agglutination of denaturated proteins.

3. The optimum acidity for precipitation in absence of electrolytes is found to be at a concentration of hydrogen-ions equal to about 3×10^{-6} normal in case of the denaturated protein of both serum and egg-white, thus confirming the figure obtained by Michaelis and Rona for the iso-electric point of serum-albumen.

4. Agglutination is greatly influenced by the presence of neutral salts. Their action is twofold:

(*a*) Alteration of the reaction of protein-containing solutions. The concentration of hydrogen-ions is lowered in acid solution and that of hydroxyl-ions in alkaline solution.

(*b*) Neutralisation or increase of the electric charge carried by the protein particles, according as the charge on the protein is of opposite or similar sign to that carried by the more potent ion of the salt.

5. In case of egg-white, agglutination of the denaturated protein is assisted by the addition of electrolytes and the range of hydrogen-ion concentration over which agglutination occurs is extended.

6. In the case of serum-proteins a similar result is obtained only when salts are present in very low concentration; in presence of more concentrated electrolytes agglutination is hindered if not altogether prevented.

7. The cause of dispersion by salts appears to be the adsorption of ions by the denaturated particles of protein. If the charge of the more potent ion is of opposite sign to their own charge they will after being first discharged, become re-charged with a sign opposite to that they originally carried. The effect is increased with higher valency.

Denaturated serum-protein is easily dispersed by electrolytes but with egg-albumen dispersion accompanied by reversal of charge was detected only after the addition of sodium citrate.

In the case of dispersion by divalent kations such as Ca^{++} we were not able to demonstrate the existence of any charge on the dispersed particles.

8. For every solution containing denaturated protein there is a critical temperature depending on the reaction and on the concentration of protein and electrolytes, below which agglutination does not take place.

9. Immediately above this critical point, rise of temperature has at first a very marked effect in enhancing the rate of agglutination; this influence, however, becomes less and less, until at temperatures far removed from the critical temperature, the effect is consistent, agglutination-rate being increased about 2–5-fold per rise of 10° C. An explanation is suggested.

10. No support is found for the conclusion both of Starke and Moll that albumen, when heated in alkaline solution, is converted into globulin; between (*a*) the dispersed protein, and (*b*) the coherent precipitate obtained on heating in alkaline and faintly acid solutions respectively, there appears to be no difference which cannot be accounted for by the different state of aggregation. Reasons are adduced for regarding the substance studied by these observers as merely heat-denaturated protein, obtained in a loose state of aggregation by the method employed.

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